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Morphology of the megalopa of the mud crab, *Rhithropanopeus harrisii* (Gould, 1841) (Decapoda, Brachyura, Panopeidae), identified by DNA barcode

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Abstract

The morphology of the megalopa stage of the panopeid *Rhithropanopeus harrisii* is redescribed and illustrated in detail from plankton specimens identified by DNA barcode (16S mtDNA) as previous descriptions do not meet the current standard of brachyuran larval description. Several morphological characters vary widely from those of other panopeid species which could cast some doubt on the species' placement in the same family. Besides, some anomalous megalopae of *R. harrisii* were found among specimens reared at the laboratory from zoeae collected in the plankton. These anomalous morphological features are discussed in terms of problems associated with laboratory rearing conditions.

Keywords *Rhithropanopeus harrisii*, Panopeidae, Megalopa, Barcode, 16S, Morphology, Anomalies.

Introduction

Currently three species of Panopeidaeare known for the Iberian Peninsula, *Panopeus africanus* A. Milne-Edwards, 1867, *Dyspanopeus sayi* (Smith, 1869) and *Rhithropanopeus harrisii* (Gould, 1841). While *Panopeus africanus* is an Iberian native species distributed from the Gulf of Cadiz (SW Spain) to the Mondego estuary (NW Portugal), the other two panopeids are introduced species. These are among the most widespread introduced brachyuran species in the world. *Dyspanopeus sayi* is native to the Atlantic coast of North America from Florida to Canada (Nizinski 2003), and has

been introduced to coastal areas of south-west England, Queens Dock, Swansea (Wales) (Ingle 1980;Clark 1986), to the French and Dutch coasts of the North Sea (Vaz et al. 2007), the Black Sea (Micu et al. 2010), and more recently to the Mediterranean Sea, Venice, the Marano and Varano lagoons, the Po River Delta (western Adriatic Sea) (Froglia and Speranza 1993;Mizzan 1995; Florio et al.2008) and to the east coast of the Iberian Peninsula (Schubart et al. 2012).The first report of a population of *R. harrisii* for the Iberian Peninsula was made by Cuesta et al. (1991) for the Guadalquivir estuary, but populations are present in many European Atlantic estuaries, as well as in some Mediterranean locations. The species has been extensively studied from several perspectives such as ecology, phylogeography and larval biology (Gonçalves et al. 1995; Forward 2009; Projecto-García et al. 2010).

Rhithropanopeus harrisii is a euryhaline crab typically associated with sheltered estuarine habitats. Connolly (1925) described its four zoeal stages and the megalopa, based on larvae reared from eggs in the laboratory. Further descriptions were provided by Hood (1962) and Chamberlain (1962), but the best illustrations of the larval stages are shown in Costlow and Bookhout (1971) (as underlined by Forward 2009). Nevertheless, all descriptions are incomplete compared to the current standard of brachyuran larval descriptions proposed by Clark et al. (1998).

Traditionally, descriptions of larvae have been accomplished from specimens cultivated in the laboratory under controlled conditions (temperature, salinity, density and absence of predators), and the specimens commonly originated from a single or sometimes from two ovigerous females. These circumstances may contribute to conceal the morphological variability of larvae that can be found in the field, a phenomenon already discussed in the literature for brachyuran larvae (Cuesta et al. 2002).

The use of molecular markers has demonstrated to be a powerful tool in providing accurate identifications for plankton specimens (Pan et al. 2008; Pardo et al. 2009; Ampuero et al. 2010; Marco-Herrero et al. 2013a). The identification of megalopae has traditionally been based on morphological characteristics, but sometimes it is impossible to get an accurate identification with this approach. In the present study we used partial sequences of the mitochondrial gene 16S as DNA barcode to identify the megalopae collected in the plankton. The 16S marker has proven to be an effective tool in studies of decapod crustaceans (Schubart et al. 2000;Porter et al. 2005; Ahyong et al. 2007), not only for the establishment of new species, but also to elucidate the taxonomic validity of closely related species (Schubart et al. 1998, 2001; Spivak and Schubart 2003).

In contrast to traditional descriptions, the megalopae of the present study were obtained from the plankton and identified by DNA barcode. Furthermore, in order to provide a definite morphological description of the megalopa stage of *R. harrisii*, comparisons were made not only with previous descriptions, but also with another set of megalopae which were reared in the laboratory from four zoeae I collected in the plankton.

Materials and Methods

Collection of the megalopae

Twenty-eight megalopae of *R. harrisii* were collected in July 2007 and four zoeae I in April 2011, all from the plankton of the Guadalete estuary (Cádiz-SW Spain) (36º35'24.09"N 6º13'46.19"W).

Rearing and description of the megalopae

All megalopae collected were preserved directly in 80% ethanol. The four zoeae I were placed in beakers containing filtered and well-aerated sea water at a salinity of 32 ± 1 ‰ and a temperature of 26 ± 1ºC.The larvae were fed with the rotifer *Brachionus plicatilis* (fed with *Nannochloropsis gaditana*). Rearing was finished when all zoeae had moulted to the megalopa instar. Megalopa descriptions were based on 10 specimens identified by DNA barcode.

To facilitate the microscopical observation of larvae structures, a digestion-stain procedure was carried out. Firstly, entire specimens were placed for about 10 minutes in a watch glass with 2 ml of heated lactic acid. Immediately afterwards, three drops of Clorazol Black stain (0.4 g Clorazol Black powder dissolved in 75 ml70% EtOH) were added to the heated solution. After 5-10 minutes, the specimen was removed from the solution and placed on a slide with lactic acid in order to proceed with the dissection of the appendages (Landeira et al. 2009).

Drawings and measurements were made using a LeicaMZ6 and Zeiss Axioskop compound microscope with Nomarski interference, both equipped with a *camera lucida*. All measurements were made by an ocular micrometer. The measurements taken were: cephalothorax length (CL) as the distance from the tip of the rostrum to the posterior margin of the cephalothorax; cephalothorax width (CW) as the maximum

114 width of the carapace. Two megalopae identified by DNA barcode were deposited at the
115 Biological Collections of Reference of the Institut de Ciències del Mar (ICM-CSIC) in
116 Barcelona, under accession numbers ICMD13121701 and ICMD13121702.
117

118 *DNA extraction, amplification and sequencing*

119 The identification of the megalopae was based on partial sequences of the 16S rDNA
120 gene. Total genomic DNA was extracted from muscle tissue from 1-2 pereiopods of
121 each megalopa, and incubated for 1–24 hours in 300 µl lysis buffer at 65ºC. Protein was
122 precipitated by addition of 100 µl of 7.5M ammonium acetate and subsequent
123 centrifugation, and DNA precipitation was obtained by addition of 300 µl isopropanol
124 and posterior centrifugation. The resulting pellet was washed with ethanol (70%), dried,
125 and finally resuspended in Milli-Q destilled water.

126 Target mitochondrial DNA from the large subunit rRNA (16S) gene was
127 amplified with polymerase chain reaction (PCR) and the following cycling conditions
128 for reactions: 2 min at 95ºC, 40 cycles of 20 s at 95ºC, 20 s at 45-48ºC, 45 s at 72ºC, and
129 5 min at 72ºC. Primers 1472 (5´- AGA TAG AAA CCA ACC TGG -3´) (Crandall and
130 Fitzpatrick 1996) and 16L2 (5´-TGC CTG TTT ATC AAA AAC AT-3´) (Schubart et
131 al. 2002) were used to amplify 540 bp of 16S. PCR products were sent to
132 NewBiotechnic and Biomedal companies to be purified and then two-direction
133 sequencing.

134 Sequences were edited using the software Chromas version 2.0. The final
135 sequences were blasted on Genbank database to get the best Blast matches for an
136 accurate identification. Sequences are accessible in Genbank under the accession
137 numbers KJ125076-KJ125077.

Results

Barcode identification

Using the BLAST utility (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the sequences obtained from the megalopae were compared with those deposited in GenBank. The sequences perfectly fit those of *Rhithropanopeus harrisii*, more specifically, no difference (100% match) was found between the 16S sequence for 546 bp and sequences of *R. hariisii* from Woodland Beach, Delaware, USA (ULLZ 3836), Genbank accession number AJ274697.

Nevertheless, three out of four megalopae reared in the laboratory from specimens collected as zoeae I in the plankton, did not show the general morphology and all setation patterns of those megalopal stage of *Rhithropanopeus harrisii* which had been directly collected in the plankton. According to the DNA barcode, however, these specimens clearly belong to the same species. We have considered these specimens as 'anomalous megalopa' and have provided an additional description of this type of larva.

Description of the megalopa

(Figs 1A-E; 2A, D, E, G; 3A, C, D; 4A-E; 5A, D, E)

Size: CL = 1.18 ± 0.05 mm; CW = 1.02 ± 0.05 mm; N= 5

Cephalothorax (Figs 1A, B): Rostrum is short and obliquely downward with 2 lateral simple setae at base, anterior end with a median triangular notch; the pedunculated

eyes with 8 small simple setae each; hepatic region swollen; one pair each of protogastric, mesobranchial and cardiac protuberances present; broader posterior part, margins setose.

Antennule (Fig. 2A): Peduncle three-segmented, with 3 shortsimple setae on first segment, 2 short simple setae on median segment and 2 short simple setae plus 2pairs of long plumodenticulate setae on distal segment; endopod unsegmented with 1 basal simple seta, 1subterminalsimple seta and 3terminal simple setae; exopod three-segmented, with 10 aesthetascs (arranged 0, 4, 6) and 4 setae (arranged 0, 2, 2 setae).

Antenna (Fig. 2B): Peduncle three-segmented with 6 setae (arranged 4, 1, 1); flagellum six-segmented with 10 simple setae (arranged0, 0, 1, 4, 3, 2).

Mandible (Fig. 2D): Palp two-segmented, with 5 terminal short plumodenticulate setae on distal segment.

Maxillule (Fig. 2E): Coxal endite with 12 plumose setae; basial endite with 16 setae (3 terminal plumodenticulate, 1 terminal sparsely plumose, 7 terminal cuspidate, 3 subterminal plumodenticulate, and 2 proximal plumodenticulate); endopod unsegmented with 1 proximal and 2 terminal simple setae; long epipodal seta present.

Maxilla (Fig. 2G): Coxal endite bilobed with 2+ 3 terminal plumose setae; basial endite bilobed with 6 + 6 sparsely plumodenticulate setae; endopod unsegmented and without setae; scaphognathite with 45-47 marginal plumose setae plus 2 small simple setae on each lateral surface.

First maxilliped (Fig. 3A): Epipod well developed, triangular shaped, with 5 long simple setae and 1 proximal plumodenticulate seta; coxalendite with 5 inner simple

183 setaeand 7 terminal plumose setae; basial endite with 1 inner +4subterminal+11
184 terminal sparsely plumodenticulate setae plus 2 terminal short simple setae; endopod
185 unsegmented with 4 short terminal simple setae; exopod two-segmented, with 5long
186 terminal plumose setae on distal segment.

187 *Second maxilliped* (Fig. 3C): Reduced epipod with 2 simple setae and 1
188 plumodenticulate seta; endopod five-segmented, with 1 simple, 2 simple, 1simple, 4
189 plumodenticulate + 1 short simple, and 3 proximal simple + 6 terminal
190 plumodenticulate setae, respectively; exopod two-segmented, with 2simple setae on
191 proximal segment and 5long terminal plumose setae on distal one.

192 *Third maxilliped*(Fig. 3D): Epipod well developed with a proximal marginal row of 6
193 plumose setae and 14 long simple setae; protopod with a marginal row of 7 plumose
194 setae and 1 simple + 3 plumose inner setae; endopod five-segmented, with 19,
195 14,6,9, and 9 setae respectively; exopod two-segmented with 5long plumose setae on
196 distal segment.

197 *Pereiopods* (Figs4A-E): Pereiopods 2-5 thin and setose, with long subteminal setae on
198 dactyli. Cheliped robust and setose without remarkable recurved spines, only
199 sometimes a small spine, never recurved.

200 *Sternum* (Figs 1D, E): Maxilliped sternites completely fused with 6 simple setae,
201 cheliped sternites with 4 or 6 simple setae each, pereiopod sternites 2-5 with 3 or 4, 2
202 or 3, 1 or 2, and 0 simple setae, respectively; sternal sutures are interrupted medially.
203 There are two forms according to setation; the most common is illustrated in Fig. 1.

204 *Pleon* (Fig. 5A): Six somites plus telson; setation as show.

205 *Pleopods* (Figs 5D, E): Biramous except uropods, present on somites2-5; endopod with
206 3 cincinuli; exopod with 10 long plumose natatory setae; uropod with 3 or 4 natatory
207 setae on distal segment.

208

209 *Description of anomalous megalopae*

210 (Figs 1F, 2C, F, H, 3B, 5B, C)

211 *Size*: CL = 1.12-1.14 mm; CW = 0.92-0.98 mm; N= 2

212 All three specimens exhibited the following deviations from the typical form:
213 cephalothorax with different shape, bearing vestiges of zoeal lateral spines, and a
214 reduced number of setae (Fig. 1F); antennular peduncle with remains of exopodal and
215 protopodal processes as spines (Fig. 2C); endopod of maxillule with a setation pattern
216 of 1,2,2,2 as in the zoeal endopod of the maxillule (Fig. 2F); endopod of maxilla with
217 setation 3,2,2 as in the zoeal maxillar endopod (Fig. 2H); endopod of first maxilliped
218 with 3 terminal long setae plus 1+1+1 long inner plumose setae (Fig. 3B); telson with 2-
219 3 terminal setae in the place of furcal arms and 1 pair of marginal setae as zoeal stage
220 (Figs 5B, C).

221

222

223 **Discussion**

224 Redescriptions of brachyuran larval stages are unusual, although they are
225 necessary when previous descriptions are brief, incomplete, inaccurate or deficient,
226 making them useless for reliable identifications. There are some cases of redescriptions

227 in the recent literature. For instance, *Aratus pisonii* (H. Milne Edwards, 1837) was
228 redescribed by Cuesta et al. (2006) considering that the previous description by Warner
229 (1968) referred to a clearly anomalous megalopa. The most recent redescription of
230 *Dyspanopeus sayi* by Marco-Herrero et al. (2013b) was necessary because the several
231 previous descriptions were brief and inaccurate and thus inappropriate for comparative
232 taxonomic studies. Correct descriptions of larval stages are needed for phylogenetic
233 studies and accurate identifications of plankton-collected specimens. In the case of *R.*
234 *harrisii*, the several previous descriptions of the megalopa from both laboratory reared
235 larval stages and from plankton-collected specimens are all incomplete and inaccurate
236 and do not meet the standard proposed by Clark et al. (1998), currently followed by the
237 majority of decapod larval morphologists.

238 Since the previous descriptions do not allow for an accurate identification of
239 plankton-collected specimens, the DNA barcode was used instead. Current molecular
240 tools ensure a correct identification of specimens collected in the field, which present
241 clear advantages over specimens which have been reared in the laboratory. In particular,
242 field-collected larvae allow for obtaining a better representation of natural
243 morphological variability compared to larvae originated from only one or two ovigerous
244 females cultured in the laboratory. In the present study the 16S sequences of the 10
245 studied megalopae, collected in the Guadalete estuary for morphological description, fit
246 at100% the 16S sequence of *R. harrisii* from Delawere (USA) deposited in Genbank.

247 The morphology of the megalopae of *R. harrisii* described in the present work
248 do not completely match the typical characters of the megalopa stages of panopeids,
249 although Martin (1984), based on zoeal morphology, included *R. harrisii* in the Group I
250 together with the majority of panopeids. Even when the classification was based on
251 megalopal features, the species was attributed to Group I (Martin 1988). The main

252 differences relate to rostrum morphology, the number of segments of the antennular
253 flagellum, and to the spinulation of the ischium of the cheliped.

254 The typical panopeid megalopa rostrum presents a remarkable spine at each
255 basal angle, called “horns” in some papers, but these are missing in *R. harrisii*. The
256 antennular flagellum of *R. harrisii* shows six segments while eight segments are present
257 in other panopeids such as *D. sayi* (see Marco-Herrero et al. 2013b) and *P. africanus*
258 (see Rodriguez and Paula1993). The number of segments of the antennular flagellum is
259 considered to be a conservative character at family level in other taxa (Cuesta 1999).
260 Finally, the absence of a remarkable recurved spine on the cheliped ischium is another
261 marked contrast to the majority of panopeids. Together with the above-mentioned
262 differences, this feature could challenge the phylogenetic position of this species. Future
263 molecular phylogenetic studies will help to resolve this question raised by the larval
264 morphology.

265 The setation patterns of maxillule, maxilla, first, second and third maxillipeds,
266 and sternum are described in the present work for the first time. As to the setation
267 pattern of the sternal plates some variability was observed, although the proportions
268 between sternites were always similar.

269 In the identification key to the megalopa stages of the Mediterranean Brachyura
270 by Pessani et al. (2004), *R. harrisii* is differentiated by bearing three long plumose
271 terminal setae on the distal segment of the uropod in contrast to “uropod exopod with
272 more than 3 setae”. Megalopae in the present study showed either three or four setae,
273 and in one case this variability occurred in the same specimen. The same variability in
274 the setation on the exopods of the uropods has already been described by Kurata (1970).

In the present work we also studied megalopae grown from zoeae I which had been collected in the plankton and raised in the laboratory. There is some evidence that the culture conditions (temperature and/or feeding) were suboptimal. The megalopae which developed under these conditions showed an anomalous morphology. This kind of anomalies has already been reported in other species, and not only for larvae raised in the laboratory (Willems 1982; Cuesta and Anger 2001), but also for larvae collected in the field (Cuesta et al. 2002). In all these cases, the anomalies referred to morphological character of the zoeal phase, such as the presence of short lateral spines in the cephalothorax and the setation patterns of maxillule and maxilla endopods. The available data suggest that morphological anomalies in the megalopa stage are the result of suboptimal environmental conditions (temperature, salinity, food), and that such deficiencies can occur not only during laboratory rearing but also in the natural environment.

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Figures captions

Fig.1 *Rhithropanopeus harrisii* (Gould, 1841).Megalopa, (A) frontal view; (B) dorsal view; (C) lateral view of the cephalothorax; (D,E) sternum; (F) anomalous megalopa, dorsal view

Fig.2*Rhithropanopeus harrisii* (Gould, 1841).Megalopa, (A) antennule; (B) antenna; (C) anomalous antenna;(D) mandible; (E)maxillule; (F)endopod of maxillule of the anomalous specimen; (G) maxilla; (H)endopod of maxillaof the anomalous specimen

Fig. 3 *Rhithropanopeus harrisii* (Gould, 1841).Megalopa, (A) first maxilliped; (B)endopod of first maxilliped of the anomalous specimen; (C) second maxilliped;(D) third maxilliped

Fig.4 *Rhithropanopeus harrisii* (Gould, 1841).Megalopa, (A) cheliped, with detail of the ischiumspine;(B) secondpereiopod; (C) thirdpereiopod; (D) fourthpereiopod; (E) fifthpereiopod

Fig. 5 *Rhithropanopeus harrisii* (Gould, 1841).Megalopa, (A): pleon, dorsal view; (B-C) telson of an anomalous megalopa; (D) uropod; (E) third pleopod